

# *Lipopeptides for vaccine development*

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Hamley, I. W. ORCID: <https://orcid.org/0000-0002-4549-0926>  
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# Lipopeptides for Vaccine Development

Ian W. Hamley\*

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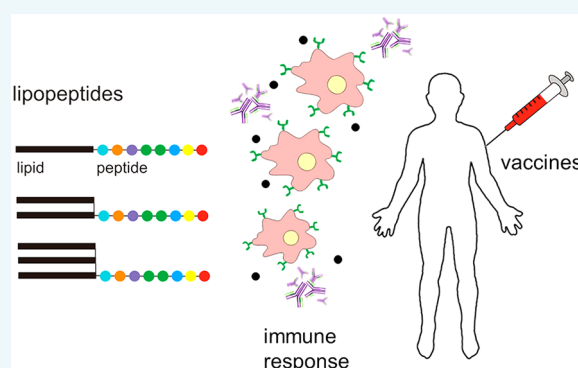
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**ABSTRACT:** The development of lipopeptides (lipidated peptides) for vaccines is discussed, including their role as antigens and/or adjuvants. Distinct classes of lipopeptide architectures are covered including simple linear and ligated constructs and lipid core peptides. The design, synthesis, and immunological responses of the important class of glycerol-based Toll-like receptor agonist lipopeptides such as Pam<sub>3</sub>CSK<sub>4</sub>, which contains three palmitoyl chains and a CSK<sub>4</sub> hexapeptide sequence, and many derivatives of this model immunogenic compound are also reviewed. Self-assembled lipopeptide structures including spherical and worm-like micelles that have been shown to act as vaccine agents are also described. The work discussed includes examples of lipopeptides developed with model antigens, as well as for immunotherapies to treat many infectious diseases including malaria, influenza, hepatitis, COVID-19, and many others, as well as cancer immunotherapies. Some of these have proceeded to clinical development. The research discussed highlights the huge potential of, and diversity of roles for, lipopeptides in contemporary and future vaccine development.



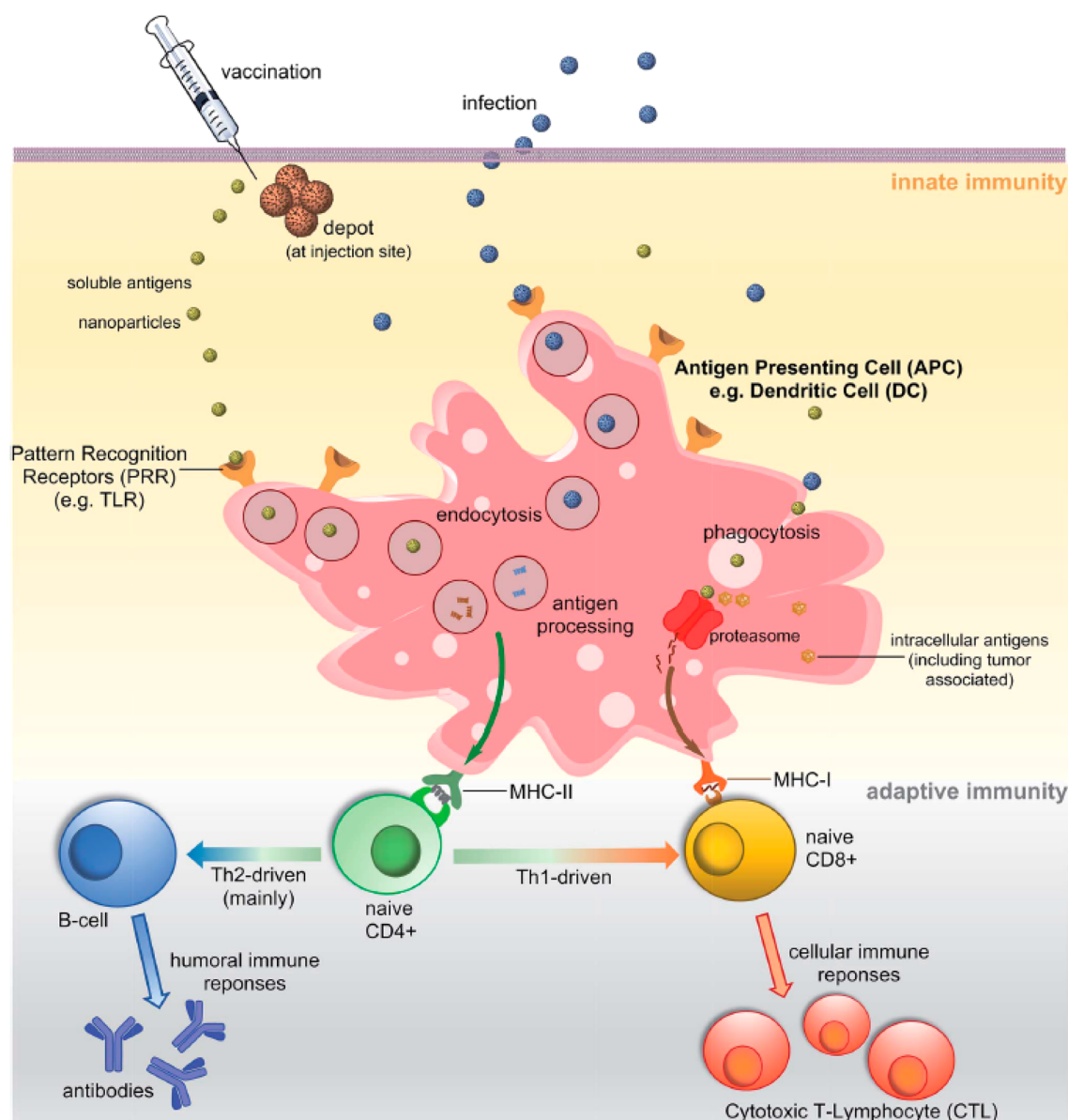
## 1. INTRODUCTION

Vaccination has been a highly successful life-saving method to prevent viral infections, since its development by Jenner in 1796 to treat smallpox, which has now been eradicated. Vaccines based on live, attenuated pathogens have been developed for many diseases; however, for other infections, such vaccines have not been successful or they are ineffective since they fail to provide long-lasting immunity (for example, influenza). In this case, vaccines that use inactivated viruses or other subunits may be created. The current COVID-19 pandemic has focused attention on the impact of viral disease on human society and already vaccines have been developed, remarkably rapidly, based on mRNA technology (the genetic material is delivered in liposomes) or the use of genetically modified (nonhuman) adenovirus carriers (which incorporate spike protein genes), or simply nanoparticle formulations containing virus spike protein subunits as well as whole inactivated viruses. Further information on this is widely available.<sup>1–6</sup> Peptides and lipopeptides are attractive in the development of vaccines, both as antigens and as adjuvants. Peptide and lipopeptide antigens can be developed based on sequences from antigenic proteins, and they can be used to stimulate cell surface receptors in a highly specific manner. Peptides offer advantages in the ease of design and preparation using automated synthesis methods, and they can also be conjugated to lipids and other molecules in the development of adjuvants (or for antigen presentation) in subunit vaccines. Conjugates of peptides and lipids, giving lipidated peptides, termed lipopeptides, are the focus of the

current Review which discusses various approaches to the synthesis and application of these molecules, a type of peptide amphiphile. Peptides are relatively inexpensive and safe to produce and can be synthesized at high purity, avoiding contaminants such as lipopolysaccharides that can be present in bioderived protein materials. A further feature of certain classes of peptide-based molecules including lipopeptides is their propensity to self-assemble into nanostructures such as fibrils, micelles, and other structures.<sup>7–13</sup> This property is of interest because it leads to presentation of bioactive peptide motifs at high density, leading potentially to improved antigen and/or adjuvant efficacy, and some examples utilizing this strategy are discussed in this Review. As yet, lipopeptide-based vaccines are not used in clinical practice, although several peptide-based vaccines have reached clinical trials and/or are currently in active development.<sup>14–19</sup> Many vaccines require formulation with adjuvants, which are biomolecules that stimulate immune responses in order to enhance the activity of a vaccine. The roles of lipopeptides as antigens and/or adjuvants is the focus of the current Review, and it includes the important class of self-adjuvanting lipopeptides, which incor-

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**Figure 1.** Cell types and other structures and processes involved in innate and adaptive immunity, which are referred to in this Review. From ref 18. Published by the Royal Society of Chemistry.

porate both antigen and adjuvant activities. This Review does not consider proteins or lipoproteins and is focused on lipopeptides. Although there is no rigorous distinction between long peptides and proteins, here we consider peptide-based systems with less than about 100 residues.

Potential treatments for cancer also include novel immunotherapies. Cancer immunotherapies include potential vaccines, monoclonal antibodies, T cell transfer therapy including CAR (chimeric antigen receptor) T cell therapy, immune checkpoint inhibitors (which modulate immune response), and immune system modulators such as cytokines (for example, interferons and interleukins). Lipopeptides have potential roles in many of these approaches; in particular, TLR (Toll-like receptor) agonist peptide-based molecules have attracted attention in cancer immunotherapies, as discussed in more detail here, and in a recent review focused on lipopeptides with applications as adjuvants for cancer vaccines.<sup>20</sup>

This Review is organized as follows. Section 2 provides a brief overview of the immune system and provides the background on

the cellular processes associated with the immune response, as well as introducing key terminology. Section 3 concerns ligated lipopeptides, in which lipid chains and peptides are coupled by ligation methods. Section 4 covers TLR agonist lipopeptides, one of the most intensely researched types of peptide amphiphiles, because some of this class provoke a strong and specific immune response, and indeed such molecules are used as model TLR agonists. Section 5 discusses lipid core peptides which have more complex architectures than those of ligated and TLR agonist lipopeptides, indeed generally offering a multi-valent presentation of peptide epitopes. Lipopeptide micelle self-assembled structures relevant to immunotherapies are discussed in section 6. Concluding remarks in section 7 close this Review.

## 2. BRIEF OVERVIEW OF THE IMMUNE SYSTEM, INTRODUCING KEY TERMS

The immune system comprises the innate and adaptive systems. The innate immune system relies on macrophages, neutrophils,

natural killer cells, dendritic cells, and others. The adaptive immune system is activated by the presentation of antigens by antigen-presenting cells (APCs) of the innate immune system. Antigen presentation involves the binding of antigen to the major histocompatibility complex (MHC), the complex then being transported to the cell surface where it is presented and is capable of recognition by a T cell receptor (TCR). This receptor binds peptides presented by class I or class II major histocompatibility complexes (also known as human leukocyte antigen, HLA, for humans) on APCs (Figure 1). Whole antigens are processed by proteolysis by APCs into short peptides (8–11 residues in length for class I MHCs and 11–30 residues in length for class II MHCs), which are presented via MHCs at APC surfaces.<sup>19</sup> TCRs that are specific for particular peptide epitopes then bind the peptide–MHC complexes, and a variety of proteins at the T cell/APC interface orchestrate expansion of clones of the T cells.

There are two types of MHC, one is involved with intracellular peptides from the cytosol (MHC class I), while MHC class II molecules bind peptides in endocytotic vesicles after internalization. The MHC-I/peptide complex activates cytotoxic T cells ( $T_c$  or killer T cells or cytotoxic T-lymphocyte CTL, a type of white blood cell) via TCRs, these cells also expressing  $CD8^+$  co-receptors (Figure 1) (here, CD refers to cluster of differentiation, a class of cell surface glycoproteins that can act as receptors or ligands). On the other hand, presentation of antigens via MHC class II is to  $CD4^+$  T cells ( $T_h$  helper T cells, another type of white blood cell which sends signals to other cells including  $T_c$  cells) (Figure 1). Only specific types of APCs such as dendritic cells, B cells, or macrophages present MHC-II at high levels, so expression of MHC-II molecules is more cell-specific than MHC-I.

The innate immune system relies on pattern recognition receptors (PRRs) to detect infecting microbes (Figure 1). PRRs are present in germline cells. They can detect a wide range of pathogens, although they lack the specificity of somatic T and B cells. Most adjuvants are ligands for PRRs. The innate immune response involves the recognition by PRRs of pathogen-associated molecular patterns (PAMPs) which include TLRs, NOD-like receptors (NLRs), C-type lectin agonists (CLRs), RIG-I (retinoic acid-inducible gene I), stimulator of interferon (IFN) genes (STINGs), and others.<sup>21–23</sup> Adjuvants based on these and other PAMPs can enhance the immune response (act as agonists) by interacting with PRRs on antigen-presenting cells. PAMPs elicit specific antigen presentation and cytokine production. The role of TLRs which are a key target for lipopeptides, as discussed later in this Review, has been reviewed extensively.<sup>24–27</sup> PRRs signal through a variety of pathways involving distinct intermediates and key transcriptional factors  $NF-\kappa B$  (nuclear factor kappa-light-chain-enhancer of activated B cells), and interferon regulatory factors IRF-3 and IRF-7 (IRFs primarily regulate type I interferons, IFNs, in the host after pathogen infection) are activated, leading to the production of cytokines and chemokines that prime and expand the immune response.

The activated adaptive immune system relies on antigen-recognizing species including T cells, B cells, dendritic cells, and antibodies (immunoglobulins). As mentioned above, the adaptive immune system produces T-helper cells which release cytokines to “help” other immune cells. T-Helper cells proliferate into differentiated Th1 cells or Th2 cells (Figure 1). The former lead to a cell-mediated response and the latter to a humoral response which refers to the production of antibodies

and antimicrobial peptides and other agents in extracellular fluid (and is also known as antibody-mediated immunity). The main effector cells of Th1 immunity are macrophages as well as  $CD8^+$  T cells, IgG B cells, and  $IFN-\gamma$   $CD4^+$  T cells. The main effector cells for Th2 are leucocytes (white blood cells) including basophils, eosinophils, and mast cells as well as B cells and IL-4/IL-5  $CD4^+$  T cells. Th1 cells produce cytokines including  $IFN-\gamma$  (interferon-gamma) and  $TNF-\beta$  (tumor necrosis factor-beta). The Th2 response leads to interleukin cytokines including IL-4, IL-5, IL-6, IL-9, IL-10, and IL-13. Th1-stimulated  $IFN-\gamma$  increases the production of IL-12 by dendritic cells and macrophages.

The activity of a vaccine may be enhanced using adjuvants, and adjuvant development is currently attracting immense interest as a means to substantially boost the performance of vaccines active against a number of diseases. Many vaccines confer humoral immunity, although adjuvants have been/are being developed to stimulate cellular ( $Th1$ ) immunity.<sup>21</sup> The most effective licensed vaccines elicit persistent T cell and B cell memory as well as long-term antigen-specific antibody responses by plasma cells.<sup>28</sup> Adjuvants are used to increase antibody production or to modulate the adaptive response. They boost the performance of vaccines, enabling a lower dose of antigen and/or a smaller number of required vaccinations. They hence increase the level of immunization within the general population that has been vaccinated and can boost immunity for groups with lowered responsiveness such as older or infant populations.<sup>21</sup> As well as generally boosting the immune response, adjuvants can be used to adjust the nature of the immune response, for example, to change the  $Th1$  versus  $Th2$  response (so-called polarization of helper T cells) or the balance of cytotoxic T cells versus helper  $CD4^+$  T cells or T cell memory as well as altering the rate of immune response and its specificity.<sup>21</sup> The type of adjuvant selected will depend on the nature of the desired  $CD4^+$  T cell response. Adjuvants should elicit a protective  $CD8^+$  response depending on the type of vaccine. Vaccines that cause direct infection of cells including viral carriers or RNA/DNA induce  $CD8^+$  immunity through the endogenous class I presentation pathway; however, other vaccines require cross-presentation.<sup>21</sup> Adjuvants have a number of roles including enhanced presentation of antigens and direct stimulation of an immune response using inactivated toxin or virus components or other immune-stimulating molecules such as bacterial lipopolysaccharides or Toll-like receptor agonists including lipopeptides, as discussed in section 4.

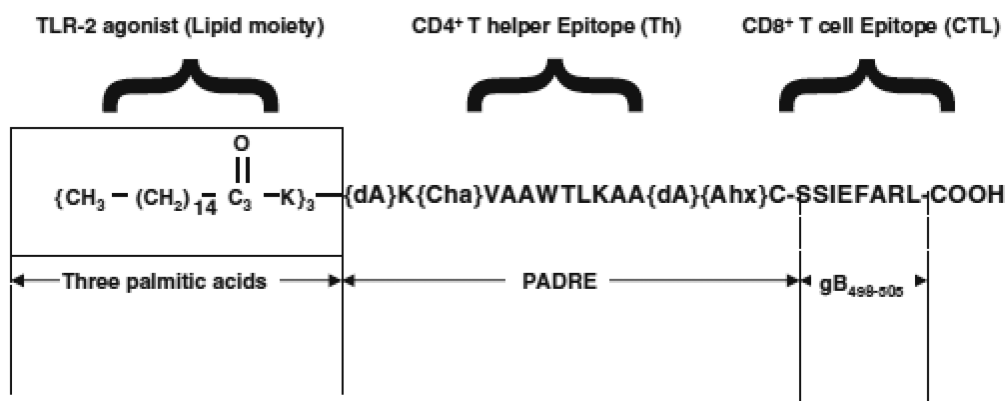
Traditionally, alum has been used as a common vaccine adjuvant, and recent reviews discuss other widely used inorganic adjuvants; see, for example, refs 21, 22, and 29. Later developments include oil-in-water emulsions such as Freund's complete adjuvant which contains heat-killed *Mycobacterium tuberculosis* (incomplete Freund's adjuvant lacks the mycobacteria) or squalene oil-in-water emulsions AS03 or MF59 (squalene is more readily metabolized than paraffin, used in Freund's adjuvants).<sup>21,22</sup> Alum and emulsion adjuvants are generally considered to have good safety profiles.<sup>21,30,31</sup> However, Freund's adjuvant contains many innate immune stimulants and can cause side serious effects; indeed, use of both Freund's complete and incomplete adjuvants was discontinued for this reason.<sup>22,31–33</sup>

Organic adjuvants such as those based on peptides, proteins, lipids, polysaccharides, and lipopeptides offer scope to tailor more specific immune responses and are the focus of considerable interest in the development of new vaccines.

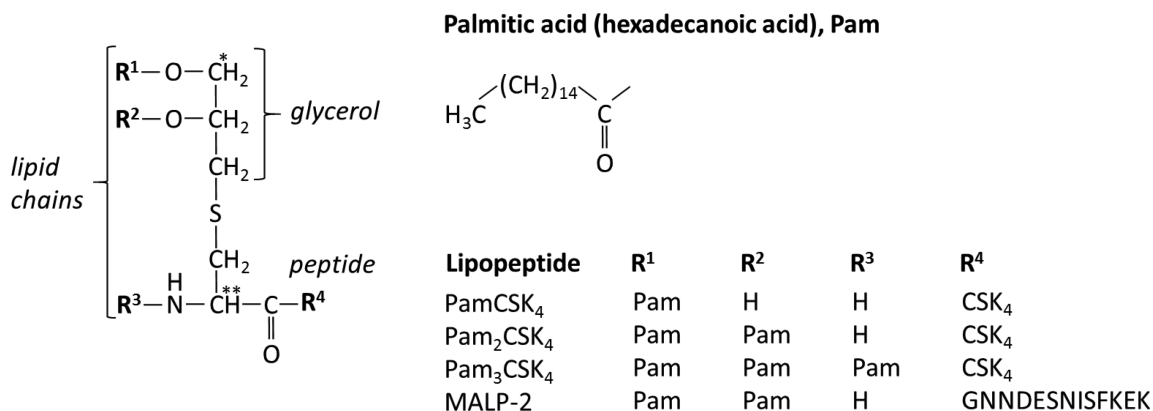


Scheme 1. Tri-Palmitoylated Lipopeptides Prepared with Dual-Function Epitopes<sup>35 a</sup>

## Th-CTL Lipopeptide (LIPO)



<sup>a</sup>The Pan DR peptide (PADRE) is a universal CD4<sup>+</sup> epitope, and gB<sub>498-505</sub> indicates the HSV glycoprotein B (gB) CD8<sup>+</sup> cytotoxic T cell immunodominant epitope. Abbreviations: dA, L-alanine; Cha, L-cyclohexyl alanine; Ahx, aminocaproic acid. Reprinted by permission from Nature Publishing Group.<sup>35</sup> Copyright 2009.

Scheme 2. General Schematic of the Structure of Pam<sub>n</sub>Cys and MALP-2 Lipopeptides in Which the Peptide Is Linked via a Cysteine-Based Unit to a Glycerol Moiety and One or More Lipid Chains<sup>a</sup>

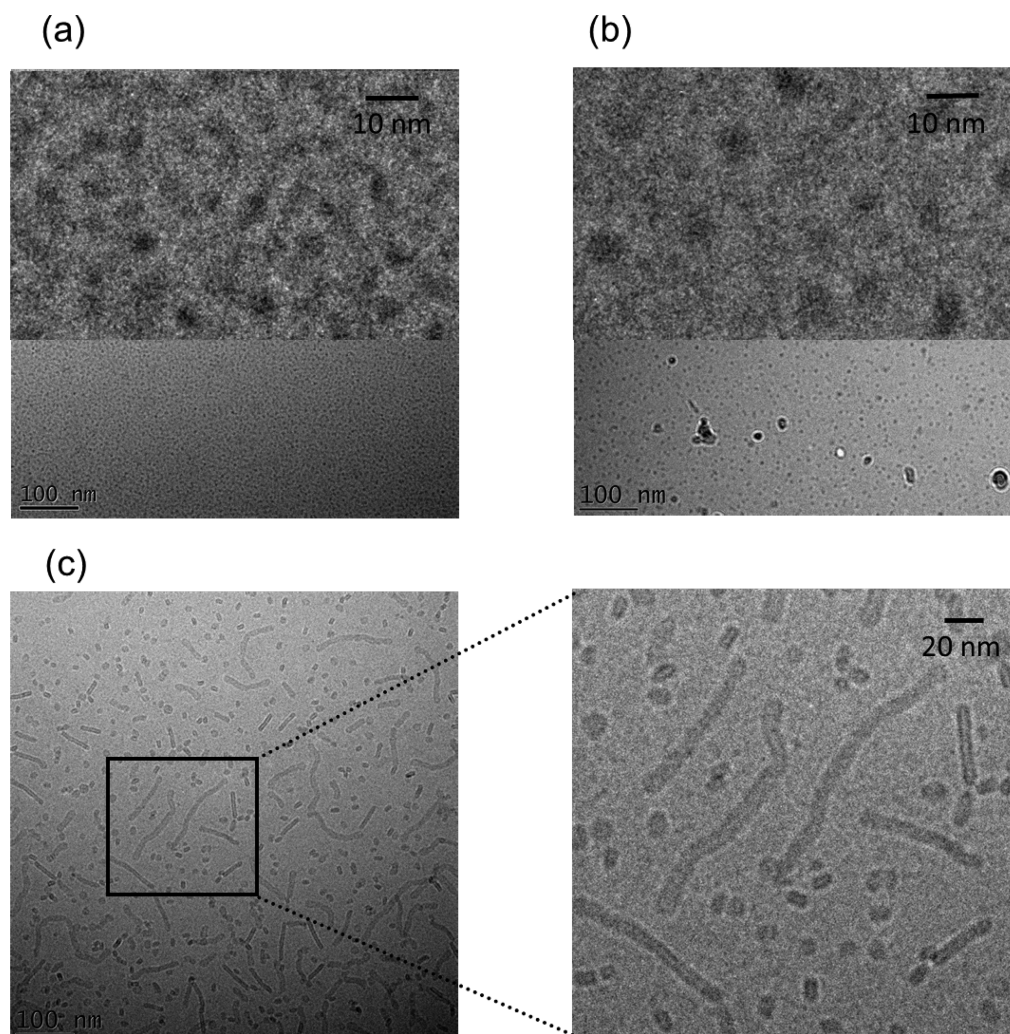
<sup>a</sup>Chiral carbon centers are indicated by \* and \*\*. Based on ref 47.

Research in the field of organic vaccine adjuvants has been reviewed.<sup>21,31,34</sup>

### 3. LIGATED LIPOPEPTIDES

A series of lipopeptides (sequences given in ref 36) containing palmitoyl chains at lysine  $\epsilon$ -amino groups has been produced as part of an HIV vaccine development program.<sup>36-38</sup> Although the conformation and self-assembly of these lipopeptides has not been examined, they show promise as vaccines, since B and T cell anti-HIV responses were detected in >85% of the vaccinated volunteers after one month, and the research proceeded to clinical trials.<sup>14,38,39</sup> In another study, palmitoylation at the lysine residue was also used to increase the immunogenicity of a series of peptides derived from four malaria parasite *P. falciparum* antigens.<sup>40</sup> Again, although information on peptide and lipopeptide conformation and ordering is not provided, these molecules generate multi-epitopic and long-lasting antigen-specific CD8<sup>+</sup> CTL responses in chimpanzees, showing potential also as human vaccines.<sup>40</sup> N-Terminal lipidation (palmitoylation) of the model antigen herpes simplex virus type 1 (HSV-1) gD<sub>1-23</sub> peptide increases uptake and maturation of dendritic cells via TLR2 and triggers Th1-dependent protective immunity.<sup>41</sup> The lipidation was performed by N-terminal

attachment of a single N<sup>ε</sup>-palmitoyl-lysine to the N-terminal lysine  $\epsilon$ -amino in the peptide using chemoselective ligation.<sup>41</sup> This method avoids problems with solid phase lipidation methods that arise due to the amphiphilicity of the lipopeptides which can complicate solution separation of the target lipopeptide.<sup>42</sup> The method can be used to prepare mixtures of lipopeptides (demonstrated with peptides based on simian immunodeficiency virus fragments and a *Clostridium tetani* sequence) and involves the site-specific introduction of a fatty acyl moiety in solution to a mixture of individually prepurified peptides.<sup>42</sup> The lipidation is based on the quasi-stoichiometric and high-yield ligation of a glyoxylyl lipid with hydrazinoacetyl peptides. This method was used to produce the HIV lipopeptide cocktails mentioned above, along with other antigen sequences.<sup>43-45</sup> A modification of this method was used to attach three N-terminal palmitoyl chains (via three K residues) to two peptide epitopes in the development of a herpes simplex virus vaccine for intravaginal delivery via the genital mucosa.<sup>35</sup> The peptide sequence includes CD4<sup>+</sup> Th and CD8<sup>+</sup> CTL sequences (Scheme 1), and the attachment of one, two, or three palmitoyl chains was motivated by the demonstrated TLR2 agonist properties of related Pam<sub>n</sub>Cys lipopeptides (discussed in section 4).<sup>35,46</sup>



**Figure 2.** Cryo-TEM images of aqueous solution structures (2 wt % lipopeptide) of (a) PamCSK<sub>4</sub>, (b) Pam<sub>2</sub>CSK<sub>4</sub>, and (c) Pam<sub>3</sub>CSK<sub>4</sub>.<sup>77</sup> Published by The Royal Society of Chemistry.

#### 4. TOLL-LIKE RECEPTOR AGONIST LIPOPEPTIDES

Lipoproteins and lipopeptides are important components of the cell wall of both Gram-negative and Gram-positive bacteria. Gram-negative bacterial membranes tend to contain lipopeptides with three lipid chains, whereas Gram-positive cell walls contain lipopeptides bearing two lipid chains.<sup>27</sup> These are heterogeneous in terms of fatty acid chain length and degree of saturation. Lipoproteins derived from Gram-negative bacteria, such as *E. coli*,<sup>49</sup> *Borrelia burgdorferi*,<sup>50</sup> *Neisseria gonorrhoeae*,<sup>51</sup> *Neisseria meningitidis* (meningococcus),<sup>52</sup> and *Porphyromonas gingivalis*,<sup>53</sup> have been shown to be TLR2 agonist triacylated lipoproteins (*N. meningitidis* lipoproteins are the basis of the vaccine Trumenba<sup>53</sup>). Lipoproteins from Gram-positive bacteria such as *Staphylococcus aureus* act on TLR2 and are diacylated derivatives.<sup>54,55</sup>

A number of lipopeptide adjuvants have been developed based on stimulation of TLRs. The core structure with one, two, or three lipid chains linked through a glyceryl cysteine linker to a peptide sequence is shown in Scheme 2, which shows the main peptide sequences that have been used and developed commercially. Peptide- and lipopeptide-based TLR agonist systems for vaccine development have been reviewed.<sup>20,27,47,56,57</sup> Tripalmitoyl-S-glyceryl cysteine (Pam<sub>3</sub>Cys) is derived from the N-terminal moiety of Braun's lipoprotein

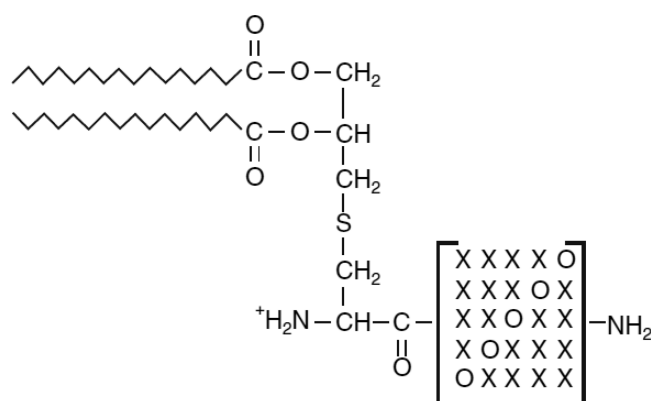
that spans the inner and outer membranes of Gram-negative bacteria. On the other hand, dipalmitoyl-S-glyceryl cysteine (Pam<sub>2</sub>Cys) corresponds to the lipid moiety of macrophage-activating lipopeptide 2 isolated from mycoplasma (bacteria which lack cell walls). It has also been reported that Pam<sub>2</sub>Cys is a more potent stimulator of splenocytes and macrophages than Pam<sub>3</sub>Cys.<sup>58</sup> It also has a negligible pro-inflammatory response, as assessed in terms of cytokine (TNF- $\alpha$ , IL-1 $\beta$ , IL-6, and IL-8) release in a study using whole human blood.<sup>59</sup> Pam<sub>3</sub>Cys activates TLR2 to produce a Th2 response, as evidenced by the production of associated effector molecules including IL-13 and IL-1 $\beta$  but not Th1-related cytokines.<sup>60</sup> Other groups found that TLR2 stimulated by Pam<sub>3</sub>CSK<sub>4</sub> promotes Th2 response,<sup>61</sup> as well as that of Th17 which is implicated in several autoimmune conditions.<sup>61–63</sup> The TLR2 activation was found to occur via other TLRs (TLR4 and TLR7/8), since cytokine production in human DCs induced by these TLRs was inhibited by TLR2.<sup>61</sup> These reports seem to be contradicted by another study, in which it is reported that Pam<sub>3</sub>CSK<sub>4</sub> and MALP-2 trigger Th1 cell function via TLR2 but do not stimulate any Th2 cell response.<sup>64</sup> The X-ray crystal structure of the Pam<sub>3</sub>Cys ligand co-crystallized with the TLR1/2 dimer has been reported,<sup>65</sup> as has the crystal structure of Pam<sub>2</sub>CSK<sub>4</sub> with the TLR2/TLR6 dimer.<sup>66</sup>

In a pioneering paper, Braun reported that a lipoprotein component of the outer membrane of *E. coli* is a specific and potent antigen.<sup>67</sup> This group had earlier identified Pam<sub>3</sub>CSSNAK as the N-terminal domain from the murein (peptidoglycan) lipoprotein obtained from the outer cell membrane of *E. coli*.<sup>68,69</sup> Shorter fragments of the SSNAK peptide were also isolated,<sup>69</sup> and synthetic lipopeptides bearing these sequences were prepared. All of them were found to be mitogenic and stimulated B lymphocytes into immunoglobulin secretion, as shown by a hemolytic plaque assay.<sup>70,71</sup> The activity of all lipopeptides bearing different peptide fragments was comparable to that of the native *E. coli* lipoprotein, although those bearing only cysteine were hardly active.<sup>71</sup> The Pam<sub>3</sub>CSSNAK lipopeptide was shown to also have adjuvant activity, as shown in a study of antibody response in sheep.<sup>72</sup> This group also compared the activity of Pam<sub>3</sub>CSSNA, Pam<sub>3</sub>CSK<sub>4</sub>, Pam<sub>3</sub>CAG, and Pam<sub>3</sub>CS, and all lipopeptides were shown to have excellent adjuvant activity.<sup>73</sup> The lipopeptide Pam<sub>3</sub>CSK<sub>4</sub> was found to be a potent antigen able to fully replace Freund's complete adjuvant (PCS) enhancing immunoglobulin production in mice.<sup>73</sup> Earlier studies on Pam<sub>3</sub>Cys-based peptides with adjuvant or antibody-stimulating properties or self-adjuvating forms have been summarized.<sup>74</sup>

Peptides and lipopeptides such as Pam<sub>2</sub>CSK<sub>4</sub> and Pam<sub>3</sub>CSK<sub>4</sub> were developed with enhanced amphiphilicity (compared to the native *E. coli* murein lipoprotein), with the additional lysine residues conferring solubility.<sup>73</sup> These molecules are agonists of TLR2 in particular and the Pam<sub>3</sub>Cys variants also with TLR1.<sup>20,58,59,75,76</sup> The peptide conformation and self-assembly properties of the mono-, di-, and tri- palmitoylated CSK<sub>4</sub> lipopeptides were compared using a combination of CD spectroscopy, cryo-TEM, and SAXS.<sup>77</sup> This revealed that PamCSK<sub>4</sub> and Pam<sub>2</sub>CSK<sub>4</sub> form spherical micelles (Figure 2a,b) in which the peptide adopts an unordered conformation. In contrast, Pam<sub>3</sub>CSK<sub>4</sub> forms a population of nanotape structures (Figure 2c), based on  $\beta$ -sheet aggregation (these disorder on heating).<sup>77</sup> The experimental findings were confirmed by later atomistic molecular dynamics computer simulations.<sup>78</sup>

Due to the potent immunogenic properties of Pam<sub>n</sub>Cys lipopeptides, much work has been undertaken to determine the structural features required for activity, as well as efforts to improve the response. Jung and co-workers prepared a range of analogues of Pam<sub>2</sub>CSK<sub>4</sub> with variation in the length of the two alkyl chains and analogues of Pam<sub>3</sub>CSK<sub>4</sub> with different *N*-acyl groups.<sup>79</sup> They also determined the activity of the synthesized lipopeptides using HEK293 cells transfected with human TLR2 and firefly luciferase reporter gene, measuring the release of IL-8 in the culture medium and NF- $\kappa$ B translocation using luciferase reporter assay. They found that a critical length of more than eight carbons in the two ester chains is required to elicit a biological response, whereas the amide (*N*-acyl) chain length is less important.<sup>79</sup> Triacylated lipopeptides with short ester-bound fatty acids such as PamOct<sub>2</sub>CSSNASK<sub>4</sub> induce no response in TLR1-deficient cells,<sup>80</sup> and Pam<sub>2</sub>CSK<sub>4</sub> and the C-terminally elongated MALP-2 derivative Pam<sub>2</sub>CGNNDESNI-SFKEKSK<sub>4</sub> (MALP2-SK<sub>4</sub>) induce cell activation in a TLR6-independent manner.<sup>81</sup> These molecules can be used as TLR-independent controls in studies of immunogenic response. This group also screened randomized Pam<sub>2</sub>Cys variants (Scheme 3) for B cell proliferation activity using murine splenic lymphocytes.<sup>82</sup> This led to the identification of two lipopeptides, Pam<sub>2</sub>CGQHMH-NH<sub>2</sub> and Pam<sub>2</sub>CSSHMH-NH<sub>2</sub>, with en-

**Scheme 3. Pam<sub>2</sub>Cys Variants Studied by Wiesmüller and Coworkers<sup>a</sup>**

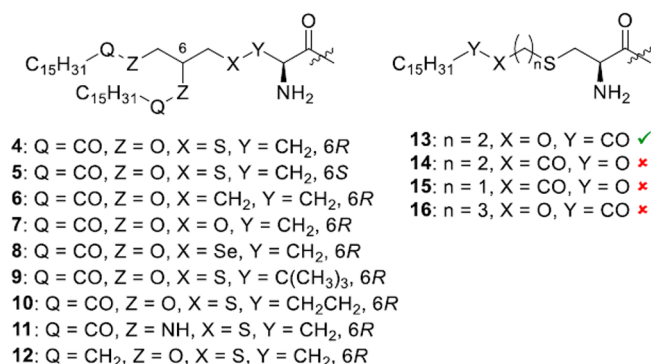


<sup>a</sup>Here X is one of the 20 standard amino acids and O is any of these amino acids except cysteine.<sup>82</sup> Copyright 2005 John Wiley and Sons Inc.

hanced activity compared to Pam<sub>3</sub>CSK<sub>4</sub>. As well as the variants shown in [Scheme 3](#), this group also investigated the effect of lipid chain length from C<sub>6</sub>–C<sub>20</sub> in Pam-Cys(Dhp)-SSNASKKKK-based [Dhp: 3,4-dihydropyran] homologues, and the lipopeptide-induced IL-8 release from HEK 293-TLR2 cells was assayed.<sup>83</sup> The activity was found to be significantly reduced for lipopeptides with lipid chain lengths shorter than C<sub>10</sub>. Ester-bound oleic acid and/or linoleic acid gave the best results in the IL-8 release assay.<sup>83</sup>

As mentioned above, a number of groups have investigated structure–activity relationships in terms of the structure of the S-(2,3-dihydroxypropyl)-L-cysteine linker present in several TLR2 agonist lipopeptides. [Scheme 4](#) summarizes the molecular structures in these studies.<sup>20</sup>

**Scheme 4. Summary of Molecular Structures from Structure–Activity Relationship Studies on TLR2 Agonist Lipopeptides Based on the S-(2,3-Dihydroxypropyl)-L-cysteine Core Present in Pam, CSK<sub>4</sub> and PamCSK<sub>4</sub>**<sup>20</sup>



Studying MALP-2 (**Scheme 2**), Takeuchi et al. reported the significantly greater TLR2 activity of the *R* stereoisomer **4** (**Scheme 4**) compared to the *S* version **5**, based on the analysis of cytokines, chemokines, or NO released by cells.<sup>84</sup> Substitution of the thioether moiety with either a methylene (**6**)<sup>85,86</sup> or oxyether (**7**)<sup>87</sup> group leads to a loss in TLR2 activity, whereas substitution of S with Se (in **8**) causes an upregulation of p38 mitogen-activated kinase (p38MAPK) in neutrophils.<sup>88</sup> The *R*-cysteinyl form of Pam<sub>2</sub>CSK<sub>4</sub> is also maximally active in reporter



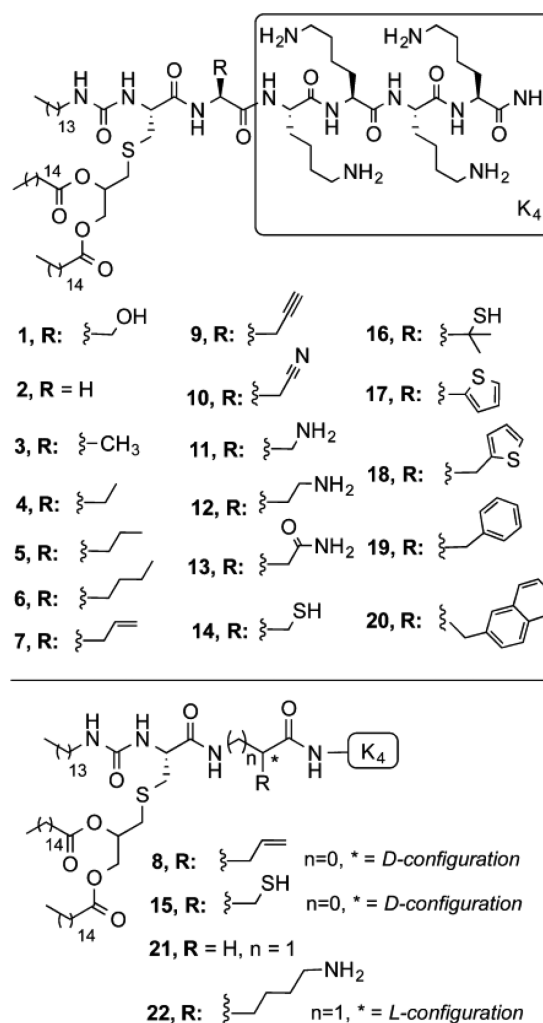
gene assays using human TLR2, as revealed in a study of the role of the highly conserved Cys residue as well as the geometry and stereochemistry of the Cys-Ser dipeptide unit.<sup>87</sup>

Changing the length of the spacer between the thioether and the  $\alpha$ -center of cysteine (**9** containing a penicillamine-based linker or homocysteine-based **10**) leads to dramatic reductions in TLR2 activity.<sup>88</sup> Substitution of the ester functionalities for amide (in **11**)<sup>89</sup> or ether (in **12**)<sup>90</sup> groups in lanthionine scaffolds strongly inhibits TLR2 activity. Compound **13** (corresponding to PamCys, Scheme 2) has pronounced TLR2 agonist activity.<sup>88</sup> However, inversion of the ester bond (in **14** and **15**) or extension of the chain between the thioether and ester groups leads (in **16**) to a significant reduction in activity.<sup>88</sup> A derivative with a thioethanol bridge in place of the thioglycerol unit retained TLR2-specific NF- $\kappa$ B induction activity.<sup>88</sup> The role of stereochemistry (*R*- and *S*-stereoisomers of the glycerol moiety) has been examined for Pam<sub>3</sub>CSK<sub>4</sub>, and it was found that the *R*-stereoisomer has greater activity,<sup>74,91</sup> similar to the case of MALP-2. This was ascribed to enhanced TLR2 triggering due to greater DC activation which stimulates CD8<sup>+</sup> T cell responses, in particular higher IL-12 secretion, and upregulates relevant markers for DC maturation.<sup>91</sup> Methylene substitution [to give 2-(palmitoylamino)-6,7-bis(palmitoyloxy)heptanoic acid (**6**)] leads to lower activity than Pam<sub>3</sub>CSK<sub>4</sub> at a given concentration, although the maximal activity obtained at significantly higher concentration is similar to that of Pam<sub>3</sub>CSK<sub>4</sub>.<sup>86</sup> The role of stereochemistry was also examined, all four stereoisomers being prepared, the 6*S* form was active in terms of radiolabeled [<sup>3</sup>H]thymidine incorporation (a cell proliferation assay) into the DNA of mice splenocytes after stimulation with lipopeptides than the 6*R* form, with little influence of the other (2*S*/2*R*) stereocenter.<sup>86</sup> The authors also noted that these lipopeptides constitute potent macrophage activators with anticancer activity: in particular, the (2*S*,6*S*) stereoisomeric form was able to induce tumor cytotoxicity.<sup>86</sup> Further details of the synthesis of the derivatives discussed in this section are provided by Brimble and co-workers.<sup>20,57</sup>

A series of Pam<sub>2</sub>CSK<sub>4</sub> analogues (Scheme 5) were prepared that contain a urea (carbamyl) linker (hence, they were termed UPam) to one lipid chain and a range of substitutions for the serine residue.<sup>92</sup> The motivation for the N-tetradecylcarbamyl substitution was the expectation that immunostimulatory activity would be enhanced, on the basis of modeling of the crystal structure of the X-ray structure of the Pam<sub>3</sub>Cys ligand co-crystallized with the TLR1/2 dimer.<sup>65</sup> Probing DC maturation, the authors observed that UPam derivatives 3–5, 7, 9, and 10 (Scheme 5), which contain small, mostly hydrophobic side chains substituting for the hydroxymethyl of 1 (UPam-Ser), show an increased potency compared to Pam<sub>3</sub>CSK<sub>4</sub> and those containing amino acids with straight alkyl chains of moderate size terminated with a polar functional group (12 and 13) also have enhanced activity (compared to Pam<sub>3</sub>CSK<sub>4</sub>). In contrast, the derivatives 8, 14, 15, 20, and 22 were found to have minimal activity.<sup>92</sup>

Lipopeptide Pam<sub>3</sub>CSK<sub>4</sub> has been investigated as an adjuvant. For example, it can elicit immunogenicity, i.e., CTL responses to *Plasmodium berghei* circumsporozoite (CS, a parasite that can cause malaria in rodents) peptide epitope SYIPSAEKI either in mixtures or by conjugation of this sequence onto Pam<sub>3</sub>CSK<sub>4</sub> (to give Pam<sub>3</sub>CSK<sub>4</sub>SYIPSAEKI), as assayed using mouse splenocytes.<sup>93</sup> In addition, a conjugate of Pam<sub>3</sub>Cys with poly(ethylene glycol) (PEG) also known as polyoxyethylene (the synthesis of the conjugate being described earlier<sup>94</sup>) was investigated as part

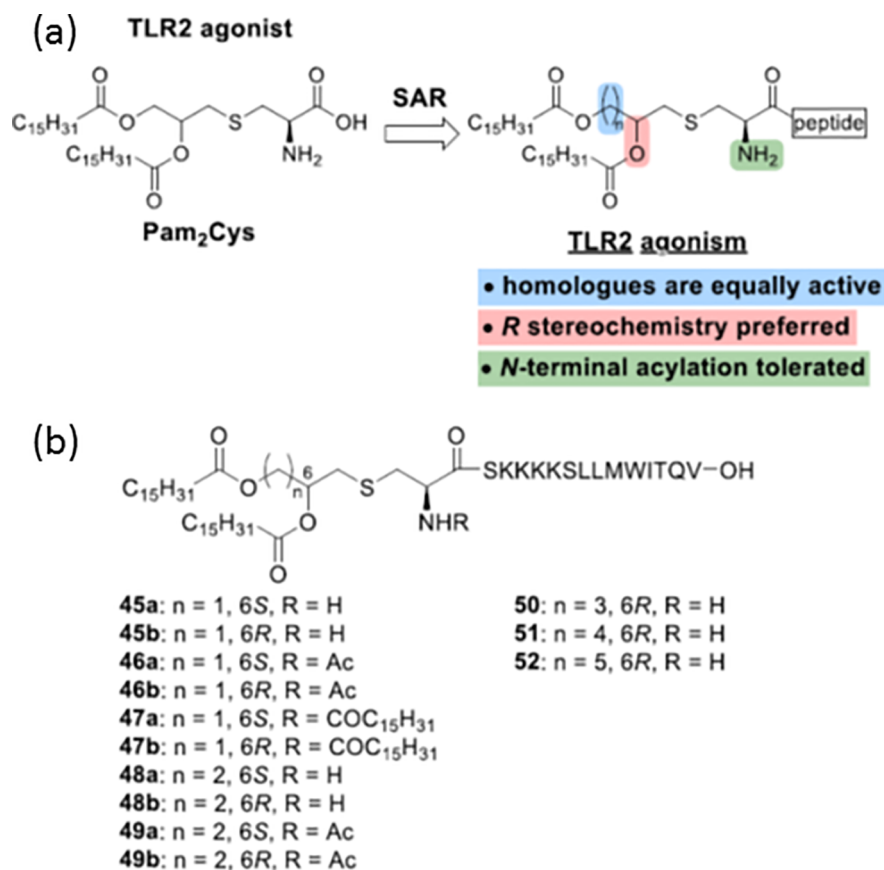
**Scheme 5. Urea-Functionalized Pam2Cys Analogues (UPam Derivatives) Studied by Willems et al.<sup>92</sup>**



of the range of formulations studied, and this conjugate mixed with CS also generated a CTL response.<sup>93</sup> The influence of the structure of Pam<sub>2</sub>Cys analogues on TLR2 agonist activity prepared as potential adjuvants for cancer vaccines has been examined.<sup>20</sup> The effects of homologation between the two ester functionalities, *N*-terminal acylation, and acyl group stereochemistry (Scheme 6) were examined in lipopeptides bearing the SK<sub>4</sub> sequence linked to an epitope from the tumor associated NY-ESO-1 protein based on the sequence SLLMWITQC (Scheme 6b).<sup>20</sup> For the bioactivity assays, HEK-Blue-hTLR2 cells were transfected with the TLR2-NF- $\kappa$ B-SEAP reporter-gene system. This system has been taken forward into commercial development by SapVax.<sup>95</sup> Pam<sub>3</sub>CSK<sub>4</sub> has also been used in the development of an HIV vaccine, by conjugation to the 32-amino-acid group-specific antigen peptide containing at least five CTL epitopes, NPPIPVGEIYKRWILGLNKIVRM-YSPTSILD.<sup>96</sup>

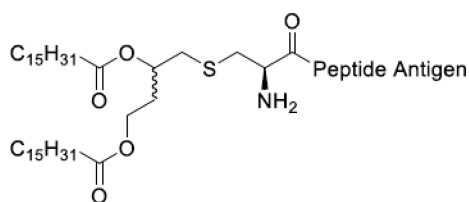
Direct thiol–ene coupling has been used to alkylate CSK<sub>4</sub> with an S-palmitoyl chain at the N-terminus.<sup>97,98</sup> In addition, as a model vaccine cytomegalovirus (CMV) ppUL83 protein sequence NLVPMVATV<sup>99</sup> was attached to produce an antigenic lipopeptide bearing the SK<sub>4</sub>NLVPMVATV sequence. Strong up-regulation of the co-stimulatory molecule CD80 on human monocytes in fresh blood samples was observed using



Scheme 6. Pam<sub>2</sub>CSK<sub>4</sub>-Based Lipopeptide Molecular Structures Studied by Brimble's Group<sup>20 a</sup>

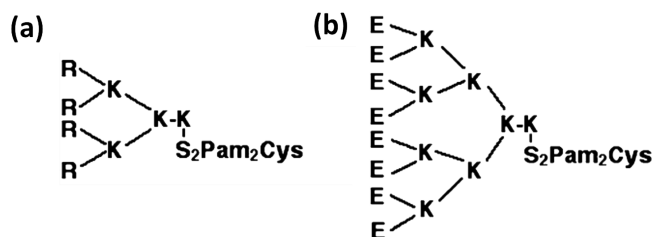
<sup>a</sup>(a) Schematic of the architecture, (b) molecular structures of specific molecules investigated.

flow cytometry, higher than that for Pam<sub>3</sub>CSK<sub>4</sub>.<sup>97,98</sup> This reaction was later studied in more detail, due to the presence of an observed byproduct (shown in Scheme 7), and conditions to

Scheme 7. Homologue of Pam<sub>2</sub>Cys Termed *homo*Pam<sub>2</sub>Cys Prepared by Brimble's Group<sup>20</sup>

obtain either mono- or bis-palmitoylated cysteine derivatives were identified.<sup>100</sup> The molecule in Scheme 7 is a homologue of Pam<sub>2</sub>Cys with an extra methyl group in the glyceryl linker and was termed *homo*Pam<sub>2</sub>Cys. It was isolated as a mixture of stereoisomers and was tested for NF-κB induction, and it showed the same activity as Pam<sub>2</sub>Cys itself.<sup>20</sup> The effect of the stereochemistry was investigated by preparing *R* and *S* enantiomers of Pam<sub>2</sub>Cys, *homo*Pam<sub>2</sub>Cys, and analogues.<sup>20</sup> Based on the NY-ESO-1 protein sequence SLLMWITQC mentioned above and the HEK-Blue TLR2 reporter system, it was found that the *R* diastereomers have activity, whereas the *S* versions show a pronounced reduction in TLR2 activity.<sup>20</sup>

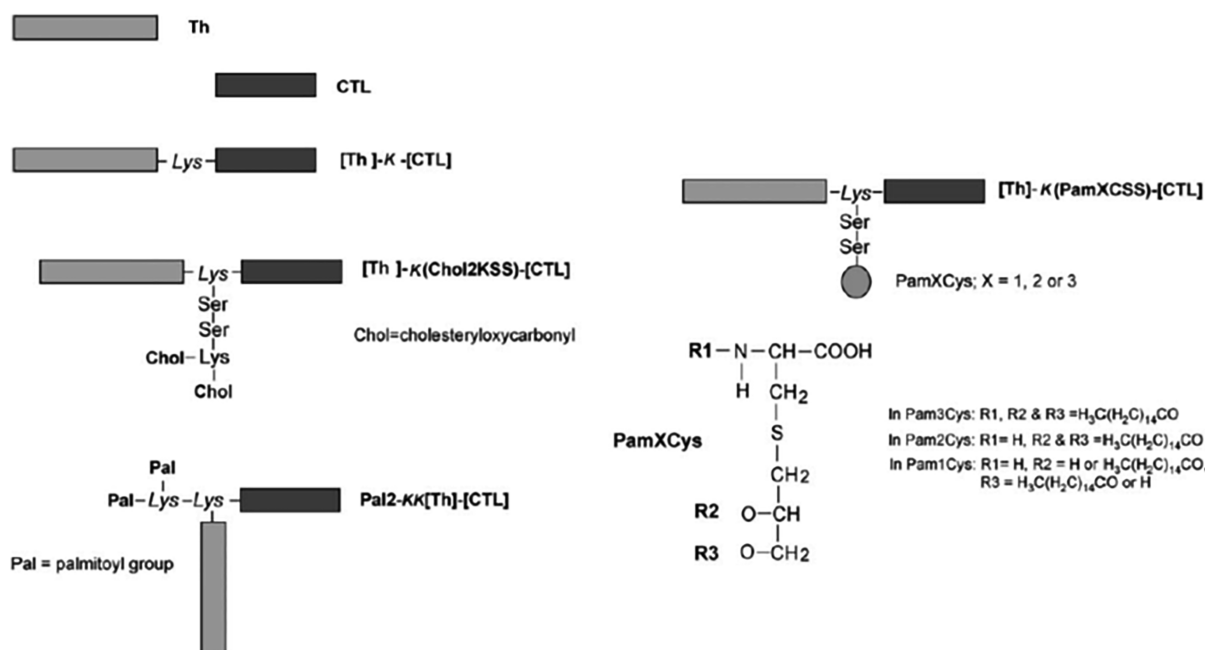
Derivatives based on Pam<sub>2</sub>Cys bearing branched highly cationic or anionic *N*-termini have been designed (Scheme 8) in order to facilitate interaction with oppositely charged soluble

Scheme 8. (a) R<sub>4</sub>Pam<sub>2</sub>Cys, (b) E<sub>8</sub>Pam<sub>2</sub>Cys<sup>101 a</sup>

<sup>a</sup>Originally published in *The Journal of Immunology*. Copyright 2011 The American Association of Immunologists, Inc.

protein antigens.<sup>101,102</sup> The binding of lipopeptide to oppositely charged antigens leads to the formation of complexes at physiological pH.<sup>101</sup> The complexes elicit a CD8<sup>+</sup> T cell immune response in mice, with concomitant pro-inflammatory cytokine production. Model antigens selected were ovalbumin (OVA) or hen egg lysozyme (HEL) with a net calculated charge of −11 or +8, respectively. The complexes were also shown to protect vaccinated mice against challenge with a live chimeric influenza virus engineered to contain the OVA CTL epitope SIINFEKL. The induced CD8<sup>+</sup> T cell response correlated with the ability of lipopeptide to facilitate antigen uptake by dendritic cells (DCs). Oppositely charged lipopeptides were more effective in DC uptake and trafficking. Substantial antibody titers were produced by vaccination with complexes composed of oppositely charged lipopeptide and protein, whereas inoculation with similarly charged constituents resulted in

Scheme 9. Architectures of Dual-Action Th- and CTL-Containing Lipopeptide Constructs Bearing Pam<sub>n</sub>Cys Units Studied by Jackson's Group<sup>104 a</sup>



<sup>a</sup>Reprinted by permission from Oxford University Press.

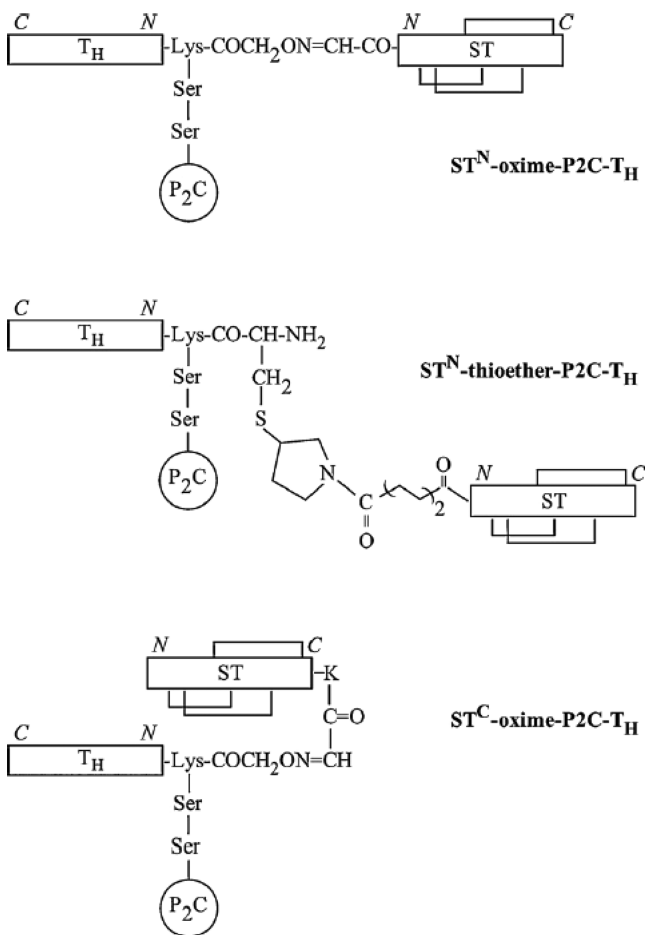
measurable but lower antibody production.<sup>101</sup> Peptide R<sub>4</sub>Pam<sub>2</sub>Cys (Scheme 8) shows a strong ability to bind OVA, leading to the formation of large complexes (ca. 400 nm radius from DLS).<sup>102</sup> Excellent adjuvant activity was noted, from measurements of antibodies in mice and cytokine production. Antibody titers were higher for the lipopeptide/OVA mixture than those elicited by OVA in the presence of alum and were comparable to those elicited by OVA formulated with complete Freund's adjuvant (CFA). The activity of the D-Arg homologue of R<sub>4</sub>Pam<sub>2</sub>Cys was also examined and was shown to stimulate similar levels of antibody production, although CD8<sup>+</sup> T cell responses (IFN- $\gamma$  secretion) were reduced.<sup>102</sup> Covalent conjugates of Pam<sub>3</sub>CSK<sub>4</sub> with OVA<sub>247–264</sub> peptide epitope DEVSGLEQLESINFEKL (or another peptide with this sequence and an A<sub>5</sub>K C-terminal extension) were prepared in a study of the TLR2 processing.<sup>103</sup> It was found that the uptake of the conjugates is TLR-independent, and inhibition of clathrin- or caveolin-dependent endocytosis greatly reduced uptake and antigen presentation of the Pam<sub>3</sub>CSK<sub>4</sub> conjugates. The lipopeptides induce a strong and specific T cell response due to the combined effects of enhanced antigen uptake, improved MHC class I antigen presentation, and dendritic cell maturation.<sup>103</sup> The Pam<sub>2</sub>Cys scaffold has been employed in the development of a range of immunogenic lipopeptides. In one example focused on the development of self-adjuvanting immunoconjugate vaccines, Pam<sub>2</sub>Cys linkers (incorporated via lysine  $\epsilon$ -amino units) were compared to Pam<sub>3</sub>Cys and control sequences just bearing lysine linkers, or other constructs with only C-terminal peptide sequences.<sup>58</sup> These bifunctional lipopeptides bear both the LHRH (luteinizing hormone releasing hormone) sequence EHWSYGLRPG and a T-helper epitope GALNNRFQIKGVLELKS (from the L chain of influenza virus hemagglutinin). The lipopeptides induce antibodies against the "self" hormone LHRH in inoculated mice, without requiring additional adjuvant. The Pam<sub>2</sub>CSS lipopeptide attached laterally via a central K residue linking the two epitopes

was highly effective, inducing high antibody titers, which were able to efficiently sterilize female mice when administered in saline by s.c. or intranasal routes.<sup>58</sup> The architecture with pendant Pam<sub>n</sub>CSS linked via the  $\epsilon$ -amine group of lysine (Scheme 9) is also most effective in stimulating DC maturation via TLR2.<sup>104</sup> This was confirmed in a study using lipopeptides with this architecture bearing a Th sequence ALNNRFQIKGVLELKS from influenza hemeagglutinin that elicits CD4<sup>+</sup> T cells and a CTL peptide epitope TYQRTRALV, i.e., NP<sub>147–155</sub>, derived from the nucleoprotein of the influenza virus which is the dominant CD8<sup>+</sup> T cell epitope recognized by BALB/c mice in all type A influenza strains.<sup>104</sup>

In another example, bifunctional Pam<sub>2</sub>Cys-based derivatives were designed as a self-adjuvanting vaccine that induces neutralizing antibodies against heat-stable enterotoxin from enterotoxigenic *E. coli*.<sup>105</sup> Three constructs were synthesized with an N-terminal helper T cell epitope (GALNNRFQIKGVLELKS, as above) and a C-terminal heat-stable enterotoxin (ST) tricyclic peptide<sup>106</sup> NSSNYCCELCCNPACTGCY (C6–C11, C7–16, and C10–C18 disulfide links) with different linker groups to the ST peptide (Scheme 10). All three compounds generated specific anti-ST antibodies; however, the low titer antibodies (using a mouse model) induced by the oxime-containing derivative demonstrated better neutralizing activity when administered via the intranasal mucosal route.<sup>105</sup> As pointed out by Moyle and Toth,<sup>47</sup> the delivery of antigens mixed with, or conjugated to, lipopeptide adjuvants has the potential to produce vaccines that are immunogenic via mucosal routes, in particular nasal or oral.

The Jackson group showed that in fact this modular approach can be extended to produce immunogenic lipopeptides bearing LHRH or acid polymerase (PA) of influenza virus with sequence SSLENFRAYV or the ST enterotoxin mentioned above linked by oxime, thioether, or disulfide units to one of three T helper cell motifs.<sup>107</sup> The first was T<sub>h</sub>(OVA) derived from OVA, with sequence ISQAVHAAHAEINEAGR, the second was T<sub>h</sub>(FLU),

**Scheme 10. Lipopeptide Conjugates Prepared by the Jackson Group with Different Linkers between a Helper T Cell Domain (Th) and the Heat-Stable Enterotoxin ST Domain<sup>a</sup>**



<sup>a</sup>The Pam<sub>2</sub>Cys (P<sub>2</sub>C) unit is attached laterally via a KSS linker. Reprinted from ref 105 with permission. Copyright 2012 Elsevier.

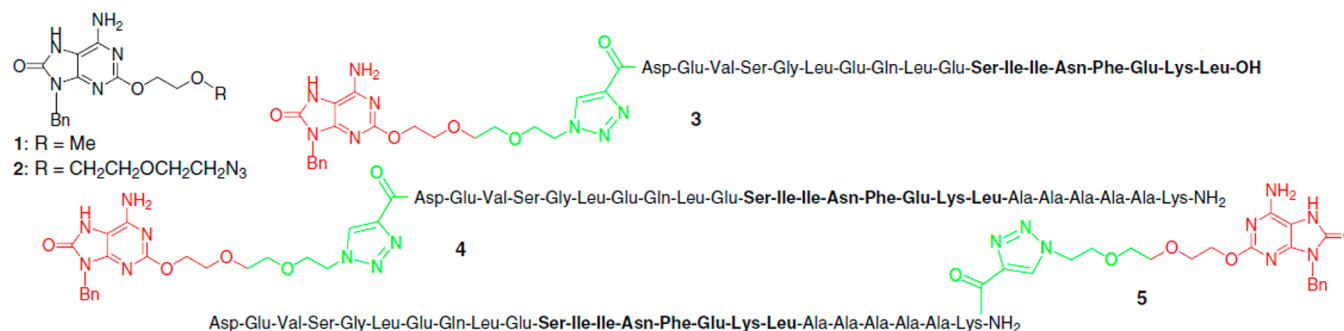
which is derived from the light chain of influenza virus hemagglutinin (sequence ALNNRFQIKGVLEKS), and the third was T<sub>H</sub>(MV), derived from the fusion protein of the *Morbillivirus* canine distemper virus (sequence KLIPNASL-IENCTKAEL). The authors note that using a thioether bond to conjugate the two components has the advantage of simpler chemistry and somewhat higher yield of product, whereas disulfide or oxime bond formation requires an additional synthesis step. Oxime bond formation, however, does have the advantage that the unwanted side products resulting from thiol groups present in cysteine residues (if present) may be avoided. The antibody and CD8<sup>+</sup> T cell responses were found to depend on both the linker group and the Th epitope employed.<sup>107</sup> In a related study, the Th sequence LNNRFQIKGVLEK derived from the light chain of influenza hemagglutinin was linked via the same linker with appended Pam<sub>2</sub>CSS (Scheme 9) to the CTL epitope, with sequence TYQRTRALV, mentioned above.<sup>108</sup> The Th domain was selected because it elicits CD4<sup>+</sup> T cells that are cross-reactive with all H3 influenza viruses and the CTL (CD8<sup>+</sup>) epitope is common to all type A influenza strains. This lipopeptide can be delivered intranasally and is able to prime lung-resident memory CD8<sup>+</sup> T cells for long-term pulmonary protection against influenza.<sup>108</sup> In another case, three Pam<sub>2</sub>Cys-based conjugates were prepared which contain a

solubilizing spacer composed of either sequences of lysine residues or polyethylene glycol (PEG).<sup>109</sup> The model proteins HEL or bovine insulin were lipidated with the three types of lipid-spacer moieties, and the immunogenicity of the lipidated species was determined in mice by measuring antibody responses. The lipopeptides contain C-terminal cysteine residues which allow for formation of either a thioether or a disulfide bond with proteins (or peptides) derivatized with MCS [N-(ε-maleimidocaproyloxy succinimide ester)] or SPDP [succinimidyl 3-(2-pyridyldithio)propionate], respectively.<sup>109</sup> The Pam<sub>2</sub>Cys scaffold has been used to attach both helper T cell Th and a target epitope that is either recognized by CD8<sup>+</sup> T cells or B cells, using a diversity of Th and target epitopes.<sup>110</sup> These examples highlight research showing that the modular ("subunit") approach to the self-adjuvant vaccine development offers considerable scope to tune the immune response. A review is available that details methods to synthesize the lipopeptides designed by the Jackson group.<sup>111</sup>

The Pam<sub>3</sub>CSS scaffold has been used along with the 16 N-terminal amino acids of the *M. tuberculosis* 19 kDa lipoprotein to give Pam<sub>3</sub>CSSNKSTTGSGETTTA which has been shown to be an agonist of RP105, a member of the TLR family that interacts with TLR2 and facilitates recognition of mature lipoproteins expressed by mycobacteria.<sup>112</sup> Mono- and dipalmitoyl analogues were also examined, as were peptide variants. These studies showed that, although the lipid moiety is required for macrophage activation, it is not a determinant of RP105 dependency. However, substitution of the T7 and T8 residues with nonpolar alanine residues led to reduced RP105 dependency.<sup>112</sup>

Like Pam<sub>3</sub>CSK<sub>4</sub>, the *M. tuberculosis* 19 kDa lipoprotein (lipopeptide) stimulates T cell proliferation via TLR2 activity, producing IFN-γ in an accessory cell-dependent manner. Sieling et al. compared the activities of these two lipopeptides along with the *Treponema pallidum* lipoprotein TP47.<sup>75</sup> *Treponema pallidum* and *Borrelia burgdorferi* are the spirochetal pathogens responsible for syphilis and Lyme disease, respectively, and lipoproteins from the membranes of these organisms elicit a strong immunoinflammatory response, as potent activators of monocytes/macrophages.<sup>113,114</sup> The OspA outer surface protein from *B. burgdorferi* contains a post-translational amino-terminal Pam<sub>3</sub>Cys lipid moiety, and this component is essential in producing an antibody response.<sup>115</sup> The full OspA lipoprotein showed significantly better adjuvant activity than Pam<sub>3</sub>CSK<sub>4</sub> in a study of intranasal delivery. Synthetic lipohexapeptides corresponding to the N termini of the *B. burgdorferi* strain B31 OspA lipoprotein (CKQNVN) and the *T. pallidum* subspecies *pallidum* 47-kDa lipoprotein (CGSSHH) prepared as glycyl cysteine derivatives (with a Pam<sub>3</sub>C motif) show similar activity, as does the unlipidated OspA hexapeptide.<sup>114</sup> The binding domains of the p19 *M. tuberculosis* 19 kDa lipoprotein to MHC class I binding motifs H-2D<sup>b</sup> and H-2L<sup>d</sup> have been identified.<sup>116</sup> Several samples from a series of PamCSS-based lipopeptides investigated elicit a CTL response, assayed as IFN-γ production from mouse splenocytes; in particular, lipopeptides which upregulated MHC-I molecules (with a H-2D<sup>b</sup> binding motif) showed this activity.<sup>116</sup>

Self-adjuvant breast cancer vaccines based on Pam<sub>3</sub>CSK<sub>4</sub> were prepared using the HER2 (human epidermal growth factor receptor-2) epitope CH401, YQDTILWKDIFHKNNQ-LALT.<sup>117</sup> Other formulations included a lipopeptide based on this sequence in a single palmitoylated peptide with a diethylene glycol linker and mixtures of this with Pam<sub>3</sub>CSK<sub>4</sub> or the

Scheme 11. Conjugates of a 2-Alkoxy-8-hydroxyadenyl Derivative (1) with Peptides (3–5) Prepared by Weterings et al.<sup>130 a</sup>

<sup>a</sup>In the conjugates, the 2-alkoxy-8-hydroxy adenine group is shown in red, while green indicates the linker region resulting from the Cu-catalyzed click cycloaddition reaction (via the azide unit in 2). The OVA peptide sequence SIINFEKL is shown in bold. Reprinted from ref 130 with permission. Copyright 2006 Elsevier.

sphingolipid  $\alpha$ -GalCer (a strong immunostimulant with antitumor properties) or Lipid A or a Pam-Th epitope QYIKANSKFIGITE (tetanus toxin-derived epitope). The conjugate self-adjuvanting vaccine produced a stronger antibody response (IgG titers from mice) than that of Pam<sub>3</sub>CSK<sub>4</sub> itself. The conjugate Pam<sub>3</sub>CSK<sub>4</sub>-CH401 conjugate self-assembles into globular aggregates, as revealed by TEM, whereas mixtures of PamCSK<sub>4</sub> and Pam-CH401 showed fibrillar co-assemblies, also observed for other mixtures containing Pam-CH401. The co-assembled structures also elicited a robust immune response.<sup>117</sup>

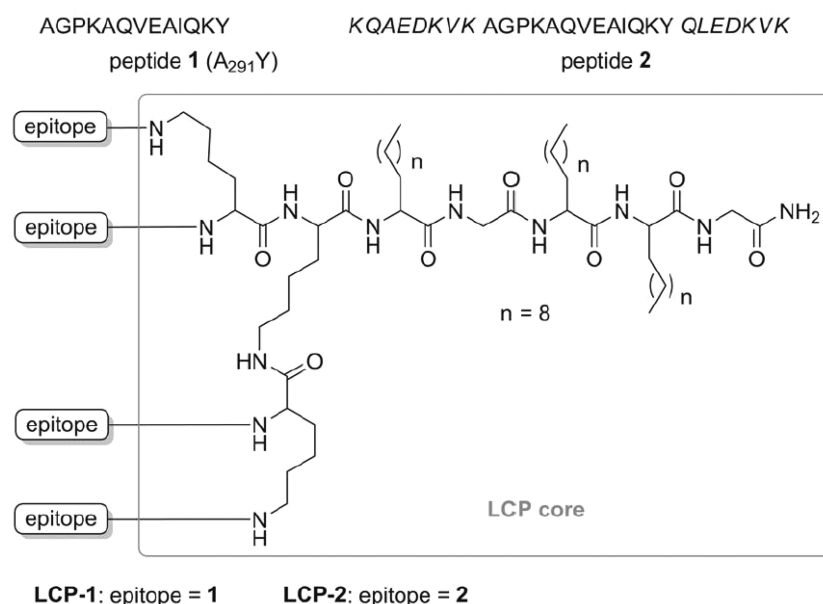
The conformational and self-assembly properties of MALP-2 have been examined in detail.<sup>118</sup> MALP-2 is a macrophage-activating lipopeptide (hence the name) originally isolated from *Mycoplasma fermentas* and contains a 13-residue peptide GNNDESNISFKEK attached to a Pam<sub>2</sub>Cys linker (Scheme 2).<sup>119</sup> Whereas three lipid chain lipopeptides such as Pam<sub>3</sub>CSK<sub>4</sub> signal via TLR1 and TLR2 as discussed above,<sup>20,48,58,59,75,76</sup> MALP-2, in common with other two-lipid-chain lipopeptides,<sup>27</sup> signals via TLR2/TLR6 heterodimers with CD36 as co-receptor.<sup>76,120,121</sup> Both MALP-2 and the constituent peptide adopt  $\beta$ -sheet conformations with different morphologies, with MALP-2 forming fibril rafts, whereas the peptide alone forms nanotape structures.<sup>118</sup> Pro-inflammatory responses in mouse lungs induced by MALP-2 and Pam<sub>3</sub>CSK<sub>4</sub> have been compared, considering the fact that the lungs have the highest expression of TLR2 receptors. The authors observed that the MALP-2-dependent induction of Tnc (tenascin C, a glycoprotein involved in inflammation) may indicate the existence of TLR2/6-specific pathways.<sup>76</sup> The recognition of MALP-2 by TLR6 cooperatively with TLR2 was noted, together with the observation that TLR6 appears to discriminate between the N-terminal lipidated structures of MALP-2 and Pam<sub>3</sub>CSK<sub>4</sub>.<sup>120</sup> Indeed, by comparing MALP-2 with the variant of MALP-2 containing three rather than two palmitoyl chains, it was inferred that three-chain lipopeptides are recognized by TLR2 while two-chain molecules (MALP-2 or lipoteichoic acid) require additional cooperation with TLR6.<sup>121</sup> In subsequent work, Mühlradt's group compared the macrophage-stimulating activities of two lipopeptides isolated from *Mycoplasma hyorhinis* and several synthetic peptides including Pam<sub>2</sub>CSK<sub>4</sub> and Pam<sub>3</sub>CSK<sub>4</sub> with MALP-2.<sup>122</sup> The assays measured nitric oxide release assay with peritoneal macrophages from C3H/HeJ mice. The isolated peptides were analogues of MALP-2 containing (in some cases) different lipid chains (and in all cases different peptide sequences), i.e., S-[2,3-bisacyl(C16:0/C18:0)oxy-

propyl]cysteinyl-GQTDNNSQSQQPGSGTTNT and S-[2,3-bisacyl (C16:0/C18:0)oxypropyl]cysteinyl-GQTN and an additional synthetic peptide S-[2,3-bis(palmitoyloxy)propyl]-cysteinyl-GQTN was examined. The GQTN motif is from the determined variable lipopeptide C (VlpC) sequence. The lipopeptides showed pM activity, i.e., were as active as MALP-2, except for Pam<sub>3</sub>CSK<sub>4</sub> which showed activity above 100 pM.<sup>122</sup> A PEGylated version of MALP-2 has been shown to produce strong humoral and cellular immune responses against enterohemorrhagic *E. coli* after intranasal vaccination in a mixture with enterohemorrhagic *E. coli* antigens.<sup>123</sup> The MALP-2 derivative was effective as an adjuvant in this mucosal (nasal) vaccine study due to its improved solubility. MALP-2 has also been used in combination with gemcitabine (a chemotherapy medication) in a phase I/II clinical trial of an immunotherapy for patients with pancreatic carcinoma.<sup>15</sup> Lipopeptides have also been derived from the 44 kDa membrane-bound lipoprotein of *Mycoplasma salivarium*, including the N-terminal lipopeptide S-(2,3-bisacyloxypropyl)-cysteine-GDPKHPKSFTEWVA-, which was used as the basis to create the synthetic analogue S-(2,3-bispalmitoyloxypropyl)-cysteine-GDPKHPKSF.<sup>124,125</sup> The latter is commercially available as FSL-1 (fibroblast-stimulating lipopeptide)<sup>125</sup> and, like other *Mycoplasma* derivatives such as MALP-2, is recognized by TLR2 and TLR6.<sup>126</sup> Variants of FSL-1 with a substitution of the C-terminal F for R or substitution of the palmitoyl lipid chains for stearoyl chains were also reported.<sup>126</sup>

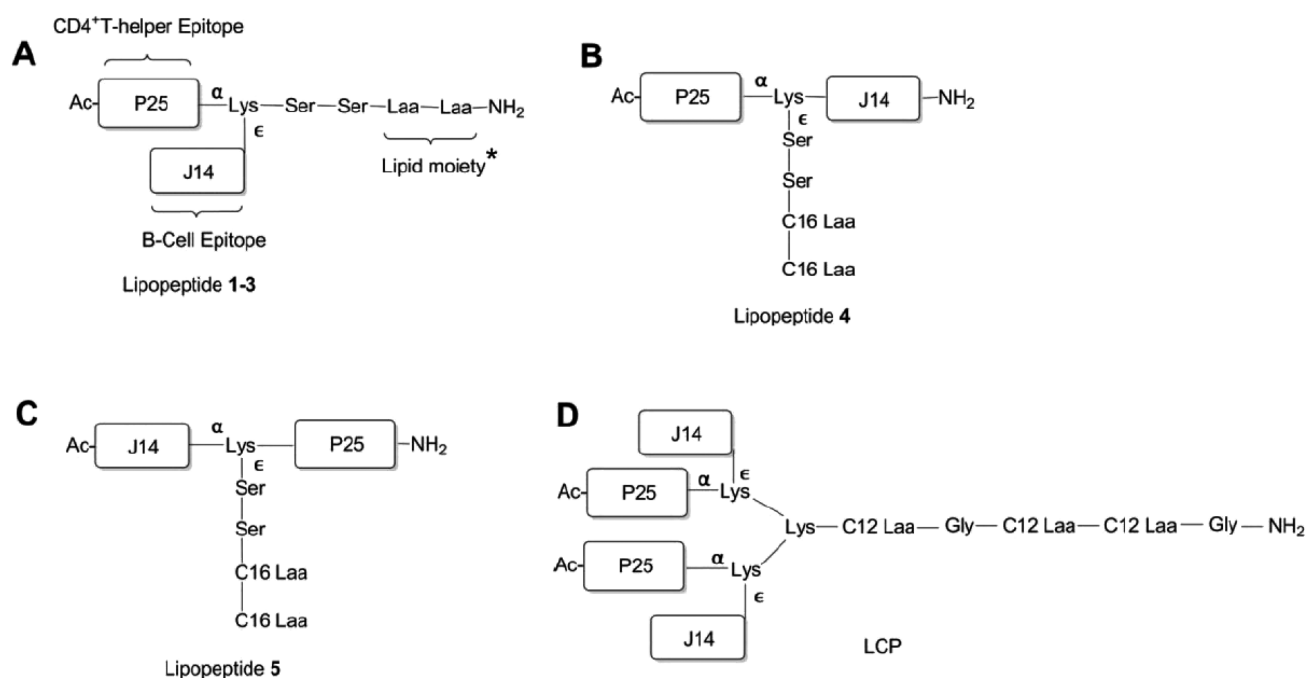
The fact that TLR2/6 agonists can reduce virus levels in the upper respiratory tract has motivated the recent investigation of the use of a Pam<sub>2</sub>Cys analogue in the treatment of COVID-19 caused by the SARS-Cov-2 virus.<sup>127</sup> Intranasal prophylactic administration of a compound INNA-051, a PEGylated Pam<sub>2</sub>Cys analogue (structure not disclosed but related to that shown in ref 109), was shown to reduce SARS-CoV-2 transmission and to provide protection against COVID-19. Stimulation of TLR2 leads to activation of the innate immune response, reduced inflammation and tissue damage, and improved local epithelial barrier function.<sup>127</sup> However, investigating respiratory syncytial virus infections, Nguyen et al. concluded that modulation of infection using Pam<sub>3</sub>CSK<sub>4</sub> is independent of TLR activation.<sup>128</sup> This was based on the observation that two structurally related lipopeptides (PamCSK<sub>4</sub> and PHCSK, a negative control for Pam<sub>3</sub>CSK<sub>4</sub> lacking a methylene group in the thioether spacer) without TLR-signaling capacity did modulate RSV infection, whereas Pam<sub>3</sub>CSK<sub>4</sub>-based TLR1/2 agonists did not.<sup>128</sup> Studying COVID-19 disease severity and its relationship to TLR2



**Scheme 12. Structure of Lipid Core Peptide (LCP) Derivatives Developed as a Peptide-Based Subunit Vaccine against Hookworm Infection<sup>132</sup>**



**Scheme 13. (A–C) Lipopeptide and (D) LCP Constructs Based on Lipoamino Acids (LAAs) and the J14 and P25 Peptide Epitopes Discussed in the Text<sup>136</sup>**



signaling, Zheng et al. identified an optimal TLR2 inhibitor by confirming the effectiveness and specificity of two different inhibitors of TLR2 signaling in bone-marrow-derived macrophage stimulated with Pam<sub>3</sub>CSK<sub>4</sub>.<sup>129</sup> Thus, Pam<sub>3</sub>CSK<sub>4</sub> has utility as a model agonist in studies on TLR2 inhibitors for COVID-19 vaccine development.

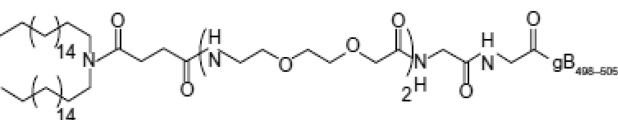
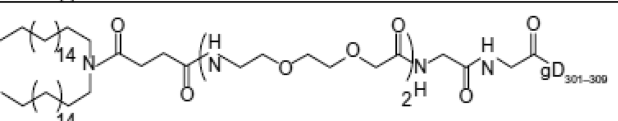
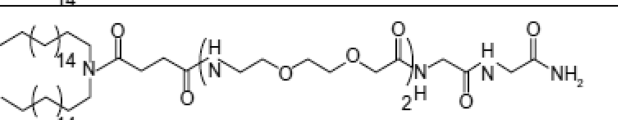
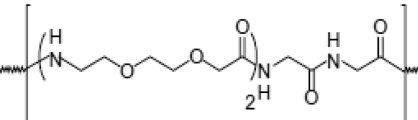
Conjugates of peptides with nucleotides have been prepared as TLR-dependent immunogens. In one example, the 2-alkoxy-8-hydroxyadenylpeptide conjugates shown in Scheme 11 were prepared.<sup>130</sup> The nucleotide component is believed to act as a TLR7 ligand. These conjugates bear the MHC class I epitope SIINFEKL from OVA. In comparison with a mixture of their

individual components, these conjugates gave rise to enhanced antigen presentation *in vitro* but were found not to be able to induce DC activation.

## 5. LIPID CORE PEPTIDES

Toth's group has developed so-called lipid core peptide (LCP) systems to deliver peptide epitopes at high density for a variety of applications, including the development of self-adjuvant vaccines.<sup>47,131</sup> Scheme 12 shows an example from a study that aimed to develop a peptide-subunit-based vaccine for hookworm infection.<sup>132</sup> The LCP in this example comprises three lipidic amino acid chains and a four-arm amine functionalized

**Table 1.** HSV Fragment Peptides gB<sub>498–505</sub> (1) and gD<sub>301–309</sub> (2) and PA Constructs Containing These Fragments (3–5) That Form HSV-Responsive Immunomodulatory Micelles, as Well as the AdOO Core Structure 6 [AdOO: 8-Amino-3,6-dioxaoctanoic Acid]<sup>a</sup>

#	Systems	Peptide epitope sequences and their amphiphilic derivatives	Mw (Da)
1	gB <sub>498–505</sub>	H-L-Ser-Ser-Ile-Glu-Phe-Ala-Arg-Leu-Amide	1,327
2	gD <sub>301–309</sub>	H-L-Ser-Ala-Leu-Leu-Glu-Asp-Pro-Val-Gly-Amide	1,307
3	(C18) <sub>2</sub> -gB <sub>498–505</sub>		1,931
4	(C18) <sub>2</sub> -gD <sub>301–309</sub>		1,911
5	(C18) <sub>2</sub> -L		1,024
6	-L-AdOO-AdOO-Gly-Gly		420

<sup>a</sup>Ref 145 (Copyright Dove Medical Press).

dendrimer. The constructs prepared bear one of two peptide epitopes shown in Scheme 12. One is a B cell peptide epitope from the apical enzyme in the hemoglobin digestion cascade, the aspartic protease Na-APR-1 termed A291Y (peptide 1, Scheme 12), while chimeric peptide 2 contains the A<sub>291</sub>Y sequence flanked on either side by helix-promoting sequences from the yeast GCN4 protein. It was found that, while A291Y alone or the chimeric peptide with or without Freund's adjuvants induce negligible antibody responses, the LCP construct incorporating the chimeric peptide induces a strong IgG response in mice. The active chimeric peptide was designed to induce the native helical A291Y epitope conformation.<sup>132</sup>

This group also developed lipopeptide-based conjugates in an effort to produce a Group A Streptococcus (GAS) vaccine.<sup>133–136</sup> The architectures of other constructs prepared by this group are shown in Scheme 13, and all contain the J14 peptide epitope KQAEKVKASREAKKQVEKALEQLEDKVK, in which the 14-residue peptide GAS M-protein C region (B cell epitope) is shown in bold, along with a universal helper T cell epitope KLIPNASLIENCTKAEL (P25). The LCP D in Scheme 13 contains two copies of J14 and P25. The vaccine of type A (Scheme 13) featuring a C-terminal palmitoyl lipid moiety, with P25 located at the N-terminus, and J14 attached to the side chain of a central lysine residue was capable of inducing the optimal antibody response after intranasal immunization in mice.<sup>133,136</sup> This group had earlier developed a LPC bearing the 88/30 GAS sequence DNGKAIYERARERALQELGC and the J8 GAS peptide sequence QAEDKVKQSREAKKQVEKALKQLEDKVK (with bold core sequence related to that in J14) in the development of a self-adjuvant intranasal vaccine targeting the GAS M protein.<sup>137,138</sup> The LCP vaccine formulation induced the elicitation of antigen-specific IgG production when administered with or without mucosal adjuvant cholera toxin B subunit, whereas cholera toxin B subunit was required

for the induction of antigens. This group also developed human papilloma virus (HPV) vaccines based on an LCP bearing four copies of a 19 amino acid long sequence QAEPDRAHYNIVTFCKCD E<sub>744–762</sub> from the HPV16 E7 protein.<sup>139</sup> The sugar D-mannose was conjugated (N-terminally) to the LCP molecules to probe the effect of targeting dendritic cell mannose receptors on vaccine efficacy. The vaccines were able to clear or reduce the size of HPV-16 associated tumors in mice, the conjugates bearing mannose causing clearance or reduction in size of tumors to a greater extent than non-mannose-containing vaccines.<sup>139</sup> LCPs can be prepared by native chemical ligation (NCL), as exemplified in a study in which four copies of the thioester-modified 88/30 GAS peptide antigen (with C to P terminal residue substitution) were ligated using a C-terminal mercaptopropionic acid leucine inker onto an LCP scaffold featuring four cysteine residues.<sup>140</sup> In another report, multiple peptide epitopes including two 88/30 GAS epitopes, the J8 sequence, and PL1 VLTRRQSQDPKYVTQRIS, an *S. pyogenes* antigen, were attached to an LCP framework using NCL.<sup>141,142</sup> Studies with mice revealed that high levels of systemic IgG antibodies were elicited against each of the incorporated peptides.<sup>141</sup> A triepitopic analogue with just one 88/30 GAS peptide attached was also prepared, and its immune response in terms of antibody titers and dendritic cell activation was assayed.<sup>143,144</sup>

## 6. LIPOPEPTIDE MICELLES

Lipopeptide micelles have been developed as nanoparticles for self-adjuvant vaccines to treat Herpes simplex virus (HSV) infection.<sup>145</sup> The micelles (presumably spherical) self-assembled from PAs containing HSV envelope glycoprotein B (gB) and glycoprotein D (gD) fragments. The critical micelle concentrations were obtained from fluorescence probe assays, and micelles were sized using dynamic light scattering. The

sequences and molecular structures of the PAs are shown in Table 1. The peptide conformation was found from CD spectroscopy to be unordered, both for single and mixed [(C<sub>18</sub>)<sub>2</sub>L-gB<sub>498–505</sub> and (C<sub>18</sub>)<sub>2</sub>L-gD<sub>301–309</sub>] peptide systems. The micelles (as well as the peptides) were shown to significantly raise *in vitro* levels of cytokines including interleukins (IL-6, IL-8, IL-17, and IL-23), macrophage inflammatory protein (MIP-2), and tissue necrosis factor TNF- $\alpha$ .<sup>145</sup>

Lipo peptides that form cylindrical micelles have been shown to generate a strong IgG1 antibody response, and these peptide amphiphiles bearing a GAS B cell antigen act as self-adjuncting vaccines for infections caused by these bacteria.<sup>146</sup> The peptide having a  $\alpha$ -helical structure was conjugated to a dialkyl (dipalmitoyl, di-C<sub>16</sub>) chain. The micelles were shown not to stimulate the pathogen-recognition receptor TLR2 *in vitro*, indicating that this is not the origin of the self-adjuncting, in contrast to the Pam<sub>3</sub>CSK4 TLR agonist used for comparison (section 4); instead, this was ascribed to the nanoparticle-mediated delivery of the peptide epitope. Thus, the lipo peptide system is non-immunogenic.<sup>146</sup> The Tirrell group also developed a micelle-forming lipo peptide containing a cytotoxic T cell epitope (SIINFEKL from OVA, discussed in section 4), linked to a di-palmitoyl (diC<sub>16</sub>) chain.<sup>147</sup> This molecule forms rod-like micelles which have a self-adjuncting property and which were shown to act not via TLR2 but instead they likely enhance uptake by DCs by anchoring of the hydrophobic tails into cell membranes. In addition, the micelles induce an immune response by acting as antigen depots and providing a high density surface presenting the epitopes and reducing degradation.<sup>147</sup> The lipo peptides induce an antitumor antibody response in mice immunized with the micelles, in particular a reduction in tumor volume, improved survival rate, and cytotoxic T cell response.<sup>147</sup>

## 7. CONCLUDING REMARKS

As the studies in this Review make clear, lipo peptides have considerable potential to play a valuable role in the development of vaccines, as antigens and/or adjuvants. There are pros and cons to the use of lipo peptides. Advantages include the ease of design and synthesis (and ability to perform large scale syntheses) of peptides along with the ability to tune conformation and self-assembly by lipidation, using established physicochemical principles. As also highlighted herein, there is scope to design active lipo peptides with a variety of architectures, i.e., configurations of lipids and peptides. In addition, lipo peptides offer the potential to tailor highly specific responses that can be achieved by precision sequence engineering and lipo peptide architecture. Other positives include the ability to prepare peptides at high purity, avoiding biological contamination and reduced allergic response. Disadvantages include the lack of the three-dimensional folded structure of a protein and hence restricted binding properties to human cells compared to actual antigens such as virus coat proteins. In addition, peptides containing native L-amino acid residues are susceptible to proteolysis and they can also have low immunogenicity. The limited stability of peptides *in vivo* can be overcome by a variety of strategies using non-natural amino acids, cyclization, etc.<sup>13,16</sup> In addition, lipidation enhances stability *in vivo*, since it confers albumin-binding properties which enable longer circulation without degradation.<sup>13,148–152</sup> Lipo peptides have as yet found limited practical use in vaccines,

although a considerable number of peptide and lipo peptide immunotherapies are in development.<sup>16–19</sup>

To be developed for practical uses, immune responses in humans should be studied as part of later stage clinical trials. Most research discussed above has focused on *in vitro* studies and those using rodent models of immune response. However, there can be significant differences in the PRRs presented in human and animal cells. The use of humanized mice or nonhuman primates as better models has thus been proposed. Also, the delivery route for many animal studies differs from the subcutaneous or intramuscular delivery used for practical vaccine delivery.<sup>21</sup> It has also been pointed out that the dose in many animal studies may differ substantially from that applicable to human use. A further limitation is the use of cell culture methods for *in vitro* studies, since these do not detect inflammatory responses generated by noncirculating tissue cells.<sup>21</sup>

The choice of antigen used together with a particular adjuvant will affect the response of DCs, for example whether a whole protein or fragment antigen such as peptide sequences discussed in this Review is selected. As highlighted in this Review, the presentation of the antigen at the surface of a self-assembled structure such as those formed by lipo peptides can also influence the immune response. In many of the cases discussed in this Review, the self-assembly and conformation of the lipo peptides has not been examined, and this is a subject worth further examination, since self-assembly may have a significant effect on bioactivity.

For successful application, lipo peptide-based adjuvants and antigens will clearly need to have an excellent safety profile. Fortunately, current adjuvanted nonlive vaccines (i.e., the type corresponding to lipo peptides) are considered to generally be insufficiently immunogenic to trigger autoimmunity. This has been well established for the widely used oil-in-water adjuvants MF59 and AS03,<sup>21</sup> and as discussed herein, some lipo peptide systems have been developed to the stage of clinical trials and do not seem to cause serious adverse reactions.

As mentioned in section 4, TLRs including those that are agonized by lipo peptides such as the Pam<sub>n</sub>Cys type are important in COVID-19 pathogenesis,<sup>153,154</sup> and this has led to a Pam<sub>2</sub>Cys-based intranasal prophylactic INNA-051 which is under commercial development.<sup>127</sup> This area is likely to be the focus of further intense research activity as the current pandemic continues to have major global effects. As outlined in this Review, lipo peptides also hold great promise in the development of vaccine treatments for many other serious infectious diseases and cancer, and thus have the potential to deliver valuable novel therapeutics based on a diversity of modes of action.

## AUTHOR INFORMATION

### Corresponding Author

Ian W. Hamley – Department of Chemistry, University of Reading, Whiteknights, Reading RG6 6AD, U.K.;  
orcid.org/0000-0002-4549-0926; Email: I.W.Hamley@reading.ac.uk

Complete contact information is available at:  
<https://pubs.acs.org/10.1021/acs.bioconjchem.1c00258>

### Notes

The author declares no competing financial interest.



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